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Tumor necrosis factor alpha blocking peptide loaded PEG-PLGA nanoparticles: Preparation and *in vitro* evaluation

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Abstract

Nanoparticles prepared from polyethyleneglycol-modified poly(D,L-lactide-co-glycolide) (PEG-PLGA-NPs) are being extensively investigated as drug carriers due to their controlled release, biodegradable and biocompatibility. The purpose of this study was to evaluate the *in vitro* characteristics of PEG-PLGA-NPs loading tumor necrosis factor alpha blocking peptide (TNF-BP). PEG-PLGA copolymer was synthesized by ring-opening polymerization of D,L-lactide, glycolide and methoxypolyethyleneglycol (mPEG) ($M_W = 5000$). Blank PEG-PLGA-NPs, with particle size within the range of 79.7 to 126.1 nm and zeta potential within the range of −12.91 to −24.55 mV, were prepared by the modified-spontaneous emulsification solvent diffusion (modified-SESD) method. PEG-PLGA-NPs uptake by murine peritoneal macrophages (MPM) was lower than that of PLGA-NPs. TNF-BP was loaded on the blank nanoparticles by electrostatic interactions, and TNF-BP loading capacity of PEG-PLGA-NPs was found to be dependent on the characteristics of blank nanoparticles, peptide concentration and incubation medium. *In vitro* release experiments showed the peptide release rate affected by the drug loading and ionic strength, and approximately 60.2% of TNF-BP released from nanoparticles still possessed bioactivity. These experimental results indicate that PEG-PLGA-NPs could be used to develop as drug carriers for TNF-BP.

Keywords: Polyethyleneglycol-modified poly(D,L-lactide-co-glycolide); Tumor necrosis factor alpha blocking peptide; Stealth nanoparticles; Spontaneous emulsification solvent diffusion method; Phagocytic uptake; Bioactivity

1. Introduction

Tumor necrosis factor alpha (TNF-α), mainly secreted by macrophages, is a cytokine with broad biological effects, such as antivirus, antitumor, immune regulation and mediating proliferation, differentiation, necrosis and apoptosis of different target cells (Slawomir and Terlikowski, 2002; Kollias and Kontoyiannis, 2002; Li et al., 2001). In proper doses, TNF-α is concerned with immune response and inflammation, which is beneficial to the host. However, in high doses it results in fever, serious inflammation and plays an important role in development of endotoxic shock and multi-organ function failure (Beutler, 1999).

In recent years, anti-TNF-α monoclonal antibodies or recombinant soluble TNF-α receptor has been developed in order to alleviate patients' sufferings from these serious side effects (Nagahira et al., 1999). However, the use of these macromolecules might induce a neutralizing antibody response after repeated administrations and cause immune complex diseases. In the previous work of our research group the TNF-α blocking peptide (TNF-BP), a cycle-seven-peptide, was obtained by screening the phage-peptide library and shown to be capable to block the biological activities of TNF-α (Ye et al., 2002; He et al., 2003). The major problem for application of TNF-BP is that it is a small peptide with molecular weight about 1 kDa so that its half-life time in blood circulation is very short.

In order to overcome the problem, drug carriers are needed to control delivery and extend half-life of TNF-BP. Some con-

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ventional particulate drug carriers have been tested for drug to provide a controlled and sustained release (Zambaux et al., 1999; Van der Veen et al., 1998). But these carriers, when given intravenously, were rapidly cleared by the cells of the mononuclear phagocytes system (MPS). At present, many studies have been concentrated on the development of the stealth nanoparticles as drug carriers, which could avoid, or at least reduce the uptake by phagocytes and prolong the time of drug in effective concentration in blood circulation. Polyethyleneglycol (PEG) modified biodegradable polymer is one of the most popular materials to prepare the stealth nanoparticles (Gref et al., 1994; Tobio et al., 1998; Avgoustakis et al., 2003; Yoo and Park, 2001). Nanoparticles prepared from polyethyleneglycol-modified poly (D,L-lactide-co-glycolide) (PEG-PLGA) are being extensively investigated as drug carriers due to their controlled release, biodegradability and biocompatibility.

Several methods have been reported for the preparation of biodegradable polymer nanoparticles, such as solvent evaporation (Gorner et al., 1999), nanoprecipitation (Bilati et al., 2005) and salting-out (Zheng et al., 2006). Among these methods, the W/O/W double emulsion solvent evaporation method is the most common used for the encapsulation of hydrophilic drug (Sanchez et al., 2003; Panyam et al., 2003). However, the disadvantage of this technique is that many hydrophilic drugs, such as proteins and peptides, may be destroyed under homogenizing procedures with high energy (ultrasonication, high pressure, or high-speed homogenization). Organic solvents are also considered to be harmful to protein or peptide. So stability of TNF-BP should be one of the most important concerns in the preparation process of loading drug on nanoparticles.

In this study, PEG-PLGA was synthesized by ring-opening polymerization. The blank nanoparticles were prepared by the modified-SESD method (Murakami et al., 1999), and the main experimental factors influencing the characteristics of nanoparticles were investigated to optimize the preparation method. PEG-PLGA-NPs were characterized by photon correlation spectroscopy (PCS) for particle size and zeta potential, transmission electron microscope (TEM) for morphological analysis, flow cytometry for the *in vitro* uptake of nanoparticles by murine peritoneal macrophages (MPM), UV spectroscopy for TNF-BP loading capacity and the *in vitro* release of TNF-BP, MTT assay for the bioactivity of TNF-BP released from nanoparticles.

2. Materials and methods

2.1. Materials

TNF-BP (M_W = 1204.4, pI = 8.6) was synthesized by CL (Xi'an) Bio-Scientific Co. Ltd. (Xi'an, China). L929 cells were kindly supplied by Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). DL-lactide (LA) and glycolide (GA) were purchased from Tianyuan Bio-materials Co. Ltd. (Fushun, China). Monomethoxypoly (ethyleneglycol) (Me-PEG, M_W = 5000, purity > 95%) and stannous octoate were obtained from Aldrich (USA). RPMI 1640, Dulbecco's modified Eagles medium (DMEM), MTT, DMSO, Penicillin–streptomycin sulfate and polyvinyl alcohol (PVA) (M_W = 23000) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Poloxamer 188 was obtained from China Pharmaceutical University. Tween 80 was obtained from Tianjin Bodi Chemical Company.

Male Kuming mice (20 ± 2 g) were obtained from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science & Technology (Wuhan, China).

2.2. Synthesis and measurement of PEG-PLGA and PLGA copolymer

2.2.1. Synthesis of copolymer

PEG-PLGA copolymer was synthesized by ring-opening polymerization (Beletsi et al., 1999). Briefly, appropriate amount of lactide, glycolide and, or methoxypolyethyleneglycol were mixed in a bottle neck flask. Stannous octoate (0.5%, w/w), as a catalyst, was dissolved in methylene chloride and added to the reaction mixture. The flask was evacuated under vacuum for 1 h and sealed. Then the polymerization was carried out at 180 °C for 4 h. After the completion of the reaction, the synthesized copolymers were purified by dissolving in chloroform and precipitating in ice-cold ether. The precipitate copolymers were filtered and dried under vacuum at 40 °C for 24 h.

2.2.2. Measurement of copolymer

Molecular weight (M_W) and molecular weight distribution (M_W/M_n) of copolymer were measured by the gel permeation chromatography (GPC) system (Waters 510 chromatographic instrument, USA) equipped with a refractive index detector. Tetrahydrofuran was used as an eluting solvent. The GPC data were calibrated with polystyrene with different molecular weights as the standards.

The Fourier transform infrared spectrum (FT-IR) was obtained from a neat film cast from the chloroform copolymer solution between KBr tablets with Perkin Elmer 1700 spectrometer (USA).

Nuclear magnetic resonance (NMR) was used to study composition of PEG-PLGA copolymers. The copolymers were dissolved in $CDCl_3$ and their ^1H NMR spectra were recorded with a Varix Mercury 300 spectrometer (USA).

2.3. Preparation and characterization of PEG-PLGA-NPs and PLGA-NPs

2.3.1. Preparation of nanoparticles

Nanoparticles were prepared by the modified-SESD method. PEG-PLGA or PLGA was dissolved in the organic solvent mixture consisting of acetone/ethanol (9:1, v/v). The copolymer solution was slowly dropped into 30 ml emulsifier solution (the preparation parameters mentioned in Section 3) under moderate stirring at room temperature. Finally, organic phases were evaporated under reduced pressure in a rotary evaporator at 40 °C.

The nanoparticle suspension was filtered through 0.45 μm millipore filter membrane (Zhongke Membrane Tech. Co., Ltd., China) to remove aggregates. The produced nanoparticles were

collected by ultrafiltrating with Millipore (MWCO 30 kDa, Millipore, USA) and washed with distilled water at least three times to remove the emulsifier. The obtained nanoparticle suspension was then used freeze-dried instrument (LGJ 0.5, Sihuan, China). The final product was stored in a vacuum desiccator at room temperature.

2.3.2. Characterization of nanoparticles

The mean size and zeta potential of nanoparticles were measured by photon correlation spectroscopy (PCS) with a Nano-ZS90 laser particle analyzer (Malvern Instruments Corp., UK) at a wavelength of 635.0 nm and a scattering angle of 90°. Samples were diluted with super pure water for PCS measurement and solution containing sodium chloride to adjust the conductivity to 50 μS/cm for zeta potential measurement. All measurements were performed at the temperature of 25 °C. Data reported represent the average of three measurements and the error was calculated as standard deviation (S.D.).

The surface morphology of nanoparticles was observed by transmission electron microscope (TEM, Tecnai G2 20, FEI Co., Netherlands). Samples for TEM observation were redispersed in super pure water, then dropped on copper grids stained with phosphotungstic acid solution (2%, w/v) and dried in air at room temperature before loaded in the microscope.

2.3.3. Storage stability of PEG-PLGA-NPs

PEG-PLGA-NPs were prepared as described above. Without any additional agents the particle samples were stored in purified water at 4 °C for a period of 4 weeks. At different time point, the particle size and zeta potential of PEG-PLGA-NPs were measured.

2.4. In vitro uptake of PEG-PLGA-NPs by murine peritoneal macrophages

The murine peritoneal macrophages (MPM) were harvested in PBS and centrifuged at 1000 rpm for 10 min and re-suspended in complete culture medium. The purification of macrophages was performed by adhesion on plate at 37 °C for 1 h, followed by wash with sterile PBS to remove non-adherent cells.

The nanoparticles loaded with Green fluorescent protein (GFP) were opsonized in 0.2 ml of mouse serum for 30 min. Then, the MPM were incubated with the opsonized nanoparticles at 37 °C for 20 min. After centrifugation (1000 rpm, 10 min), the cells were separated from free nanoparticles and washed with PBS for three times. Finally, the fluorescent intensity of nanoparticles-phagocytosed cells was analyzed by a FACScan flow cytometry (Becton Dickinson, USA).

2.5. Preparation of TNF-BP loaded PEG-PLGA-NPs

Nanoparticle suspension of defined concentration was incubated in defined amount of TNF-BP solution at 4 °C for 14 h. After the period of incubation, nanoparticle suspension was fractionated by ultrafiltration with Millipore, and obtained TNF-BP loaded nanoparticles was washed three times with distilled water by repeated ultrafiltration.

2.6. Determination of TNF-BP loading capacity of PEG-PLGA-NPs

The unloaded TNF-BP was removed from the peptide loaded nanoparticles by ultrafiltrating with Millipore. Peptide content was measured at 280 nm by UV spectroscopy (Pharmacia Biotech 4300, USA). The amount of peptide loaded on nanoparticles was calculated indirectly by determining the total amount of peptide added and the amount of peptide unentrapped.

Entrapment efficiency represented the percentage of peptide entrapped with respect to the total peptide added for nanoparticles. Drug loading represented the amount of peptide entrapped per unit weight of nanoparticles.

2.7. In vitro release studies

TNF-BP loaded nanoparticles (40 mg) were incubated with distilled water, or phosphate buffer solution (6 ml), stirring (100 rpm) with a magnetic stirrer at 37 °C in water bath. Supernatants (300 μl) were taken at different time intervals and ultrafiltrated with Millipore. The precipitated particles were re-suspended in fresh solution and returned to the incubation medium. The ultrafiltrated solution containing released TNF-BP was then analyzed by UV spectroscopy to determine the percentage release of the drug from the nanoparticles.

2.8. Evaluation of the bioactivity of peptide release from PEG-PLGA-NPs

The anti-cytotoxic effect of TNF-BP was analyzed by the MTT method. L929 cells were adjusted to a density of 2×10^5 cells/ml. 2×10^4 cells/100 μl per well were seeded in a 96-well microplate and incubated in DMEM containing 10% fetal calf serum (FCS) at 37 °C, 5% CO_2 overnight. One milliliter of TNF-BP (20 μg/ml) released from nanoparticles at different time points was pretreated with 1 ml of TNF (200 U/ml) for 1 h. Then, 20 μl of the mixture was added to the cells/well in the 80 μl serum-free RPMI 1640 medium containing actinomycin D (1.0 μg/ml) and incubated for an additional 12 h. Viability of cells was determined by staining for 4 h with 0.5 mg/ml of MTT, followed by lysis of cells with 0.1 ml of dimethyl sulfoxide. The absorbances were measured at 570 nm on an ELISA microplate spectrophotometer (Bio-Rad 550, USA).

2.9. Statistical analysis

The experiments were repeated three times and the results were expressed as mean ± S.D. Statistical analysis was done using two-tailed Student's *t*-test. In all cases, $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Measurement and characterization of PEG-PLGA copolymer

PEG-PLGA and PLGA copolymer were synthesized by ring-opening polymerization. The M_W and molecular weight dis-

Table 1
GPC results of PLGA and PEG-PLGA copolymer

Copolymer	M_W (Da)	M_W/M_n
PLGA	29000	1.96
PEG-PLGA	38000	2.14

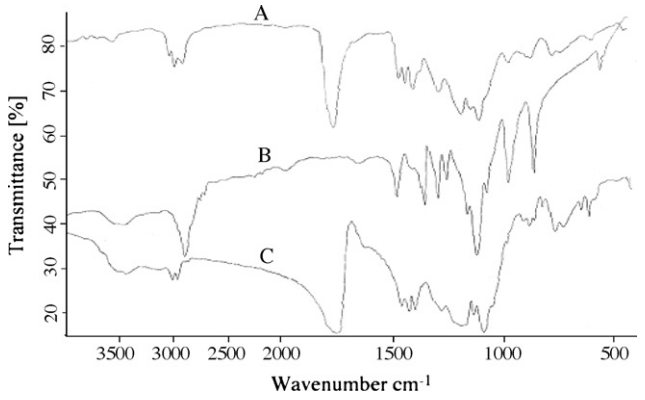

Fig. 1. FT-IR spectra of PEG-PLGA (A), mPEG (B) and PLGA (C).

tribution (M_W/M_n) of the copolymers were determined by GPC (Table 1). The copolymers were synthesized at high temperature, and the M_W of the copolymers was not easy to control. So the molecular weight distribution (M_W/M_n) was wide.

The FT-IR spectrum of PEG-PLGA, PLGA and mPEG were shown in Fig. 1. The absorption peak at 2900–3000 cm^{-1} was corresponding to the C–H stretch of CH_3. The strong absorption peak at 1760 cm^{-1} belonged to –C=O stretch, indicating the formation of the copolymer. The absorption peak at 1090–1170 cm^{-1} was attributed to C–O stretch. The comparison of FT-IR spectrum of three polymers confirmed that the reaction among lactide, glycolide and monomethoxy polyethyleneglycol (mPEG) had been done. The broad adsorption peak at 3500 cm^{-1}, –OH stretching, was shown in the spectrum of mPEG and PLGA, however, it was practically eliminated from the spectrum of PEG-PLGA.

The composition of PEG-PLGA was determined from the ^1H NMR spectrum (Fig. 2). The multiplets at 5.13 and 4.78 ppm were corresponding to the lactic acid proton (–O–$CH^*(CH_3)$–CO–) and the glycolic acid protons (–O–$CH_2{}^*$–CO–), respectively. The large peak at 3.64 ppm came from the ethylene oxide protons (–O–$CH_2{}^*$–$CH_2{}^*$–). The peaks at 1.57 ppm were attributed to the –$CH_3{}^*$ of the lactic repeat units of the copolymer.

3.2. Characterization of PEG-PLGA-NPs

The blank nanoparticles were prepared by the modified-SESD method. The main preparation parameters, such as the type of emulsifier, emulsifier concentration and copolymer concentration, were optimized in order to obtain the nanoparticles with the desired characteristics.

Previous studies (Goppert and Muller, 2005) found that positively charged particles adsorbed preferentially proteins with isoelectric point (pI) < 5.5 and the negatively ones preferentially proteins with isoelectric point (pI) > 5.5. In the case of PEG-PLGA nanoparticles, their surfaces possess negative charge. Below the pI, the net charge of TNF-BP (pI = 8.6) molecules is positive. Consequently, TNF-BP molecules could be attached to the surface of PEG-PLGA nanoparticles by electrostatic interactions (Fig. 3). Furthermore, the hydrophobic domains of peptide might absorb to the surfaces of hydrophobic nanoparticles through hydrophobic interactions (Jung et al., 2002).

3.2.1. Influence of the type of emulsifiers

According to the previous study (Schubert and Muller-Goymann, 2005), the emulsifier plays a very important role in the preparation of nanoparticles, which significantly affected nanoparticle characteristics, including particle size, polydispersity index and zeta potential. Based on above considerations, Poloxamer 188, Tween 80 and PVA, with different

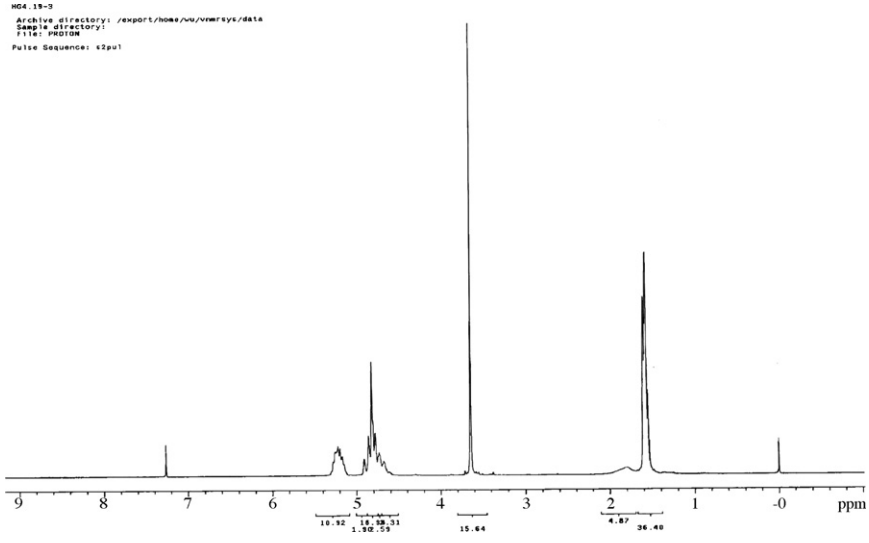

Fig. 2. ^1H NMR spectrum of PEG-PLGA copolymer.

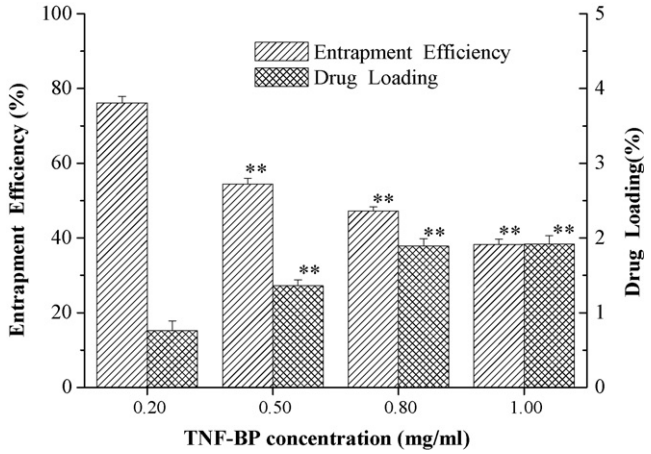

Fig. 3. Entrapment efficiency and drug loading of TNF-BP on PEG-PLGA-NPs with increasing peptide concentration. The results were expressed as mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared with the aqueous solution of 0.2 mg/ml TNF-BP.

hydrophilic–lipophilic balance (HLB), were used as the emulsifier to prepare the blank nanoparticles (Table 2). At the emulsifier concentration of 0.5% (w/v), the particle size was the lowest and the zeta potential absolute value was the highest when Tween 80 was employed.

TNF-BP entrapped on the blank PEG-PLGA-NPs was the lowest and only around 31.02% for PVA, corresponding to drug loading was 0.78 ± 0.05%. The entrapment efficiency reached 49.61 ± 1.98% for Tween 80, corresponding to drug loading was 1.22 ± 0.06%. Peptide adsorption was mainly affected by both the particle size (surface area) and the surface charge of nanoparticles. On the other hand, the different amount of the emulsifier adsorbed on the nanoparticles for dissimilar emulsifier might also affected the loading capability. In order to ensure a stable nanoparticle system and higher drug loading, Tween 80 was used in latter experiments.

3.2.2. Influence of the emulsifier concentration

The influence of the emulsifier concentration on particle size and zeta potential of blank PEG-PLGA-NPs was examined (Table 3). It showed no distinct difference in particle size by varying Tween 80 concentration from 0.2 to 1.0%. However, larger particles were observed at a concentration of 1.5%, resulting probably from a higher viscosity of the aqueous phase and consequently larger droplets within the emulsion. Negative zeta potential was decreased along with increasing the concentration of Tween 80, which might be attributed to the nonionic properties of Tween 80 overlaying the surface of nanoparticles.

As seen in Table 3, entrapment efficiency, as well as drug loading, was reduced as the concentration of Tween 80 increased from 0.2 to 1.5% (w/v). The low concentrations of Tween 80 had no great influence on the particle size, and a constant surface area could be assumed for nanoparticles. Therefore, the amount of TNF-BP adsorbed on the surface of nanoparticles was mainly affected by the zeta potential. The zeta potential of nanoparticles had no significant variance when the concentration above 1.0%. The reason that the entrapment efficiency decreased at the concentration of 1.5% may be the increased particle size made the surface area decrease.

3.2.3. Influence of the polymer concentration

The influence of polymer concentration on PEG-PLGA-NPs characteristics was shown in Table 4. The particle size showed a constant increase with the increase of copolymer concentration in the organic phase. The augment of particle size was due to an increase in viscosity of the organic phase during nanoparticles preparation and a reduction in contribution of acetone spontaneous diffusion to aqueous solution when a higher concentration of copolymer was employed. At the same time, high residual emulsifier might be coated on the surface of nanoparticles, as indicated by a remarkable zeta potential drop from −24.55 ± 3.50 to −12.91 ± 2.04 mV.

The entrapment efficiency was enhanced from 54.40 ± 1.56 to 64.29 ± 1.97% when the PEG-PLGA concentration was ele-

Table 2
Influence of several emulsifiers on PEG-PLGA-NPs characteristics

Emulsifier	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading (%)
PVA	126.1 ± 18.6*	0.208 ± 0.023**	−16.71 ± 4.12	31.02 ± 1.87**	0.78 ± 0.05**
Poloxamer 188	103.3 ± 9.4*	0.147 ± 0.015*	−14.25 ± 2.74	43.85 ± 1.23*	1.09 ± 0.04*
Tween 80	79.7 ± 8.2	0.113 ± 0.006	−19.73 ± 3.11	49.61 ± 1.98	1.22 ± 0.06

The preparation parameters: PEG-PLGA (40 mg), organic solvent mixture (5 ml), aqueous phase (0.5 % emulsifier, w/v, 30 ml), TNF-BP (1 mg). The results were expressed as mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Tween 80.

Table 3
Influence of the emulsifier concentration on PEG-PLGA-NPs characteristics

Emulsifier (%, w/v)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading (%)
0.2	80.2 ± 7.7	0.091 ± 0.009	−24.55 ± 3.50	54.40 ± 1.56	1.36 ± 0.05
0.5	79.7 ± 8.2	0.113 ± 0.006*	−19.73 ± 3.11	49.61 ± 1.98*	1.22 ± 0.06*
1.0	86.4 ± 10.5	0.068 ± 0.006*	−15.45 ± 3.53*	40.27 ± 1.23**	1.01 ± 0.04**
1.5	97.1 ± 9.4	0.046 ± 0.003**	−14.14 ± 0.80**	34.82 ± 1.71**	0.87 ± 0.05**

The preparation conditions see Table 2. The results were expressed as mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0.2% (w/v) Tween 80.

Table 4
Influence of copolymer concentration on PEG-PLGA-NPs characteristics

Copolymer (mg/ml)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading (%)
8.0	80.2 ± 7.7	0.091 ± 0.009	−24.55 ± 3.50	54.40 ± 1.56	1.36 ± 0.05
12.0	82.9 ± 8.3	0.085 ± 0.005	−19.41 ± 3.28	64.29 ± 1.97**	1.07 ± 0.03**
16.0	100.3 ± 10.6	0.099 ± 0.011	−13.04 ± 1.86**	57.88 ± 1.63**	0.72 ± 0.02**
20.0	108.4 ± 13.4*	0.098 ± 0.009	−12.91 ± 2.04**	60.67 ± 2.12**	0.61 ± 0.02**

Aqueous phase (0.2% emulsifier, w/v, 30 ml), the other preparation conditions see Table 2. The results were expressed as mean ± S.D. ($n = 3$). *$P < 0.05$, **$P < 0.01$ compared with 8.0 mg/ml PEG-PLGA in organic phase.

vated from 8.0 to 12.0 mg/ml, and The PEG-PLGA concentrations above 12.0 and up to 20.0 mg/ml resulted in a slight reduction in entrapment efficiency. In contrast, the drug loading tended to decrease with the PEG-PLGA concentration range from 8.0 to 20.0 mg/ml. One explanation is that an increase of PEG-PLGA concentration might be indicated the enhancement of the amount of nanoparticles. Moreover, a high copolymer concentration in organic phase resulted in the increase of the particle size and the decrease of surface charge of nanoparticles that would affect the amount of TNF-BP adsorbed on the surface of nanoparticles. Therefore, the appropriate copolymer concentration was important to ensure an improvement in entrapment efficiency and drug loading of nanoparticles.

3.2.4. Influence of the TNF-BP concentration

To assess the influence of TNF-BP concentrations on the loading capacity of nanoparticles, PEG-PLGA-NPs (40 mg), formulated under identical conditions, were incubated with 2.0 ml different concentrations of TNF-BP solution. As seen in Fig. 3, the higher peptide concentration induced the lower entrapment efficiency. The entrapment efficiency reached 76.13 ± 1.77% when 0.20 mg/ml TNF-BP was used. Whereas only 38.32 ± 1.35% was entrapped for 1.00 mg/ml TNF-BP. However, if we take into account, not entrapment efficiency, but drug loading which increased from 0.76 ± 0.13 to 1.89 ± 0.10% with increasing TNF-BP concentration from 0.20 to 0.80 mg/ml. Beyond 0.80 mg/ml, no further increase in drug loading occurred. The results illustrated that the higher the peptide concentration is taken, the more the peptide molecules are likely to be adsorbed. When the peptide concentration reach a saturation limit, all the nanoparticles surfaces were taken up. Above this value, drug loading would keep constant.

3.2.5. Influence of the incubation medium

The amount of TNF-BP loaded versus the different incubation medium was investigated as well (Fig. 4). At the same ionic strength, the alteration of pH in phosphate buffer solution could change the amount of TNF-BP loaded. The nanoparticles surface should be too poor charge to absorb peptides when adjusted the pH of aqueous is close to the p*I* (8.6) of TNF-BP. As expected, the entrapment efficiency was found to be from 26.63 ± 1.15% at pH 6.0 to 14.80 ± 0.74% at pH 8.0, corresponding to drug loading drop from about 0.67 ± 0.03 down to 0.37 ± 0.02%. In addition, the behaviors of loading drug on nanoparticles were dramatically dependent on the ionic strength of incubation medium. A maximum entrapment efficiency, up to 54.40 ± 1.56%, was observed in distilled water for a low ionic strength. Where drug loading was 1.36 ± 0.08%. This result was consistent with the fact that high ionic strength shields the charges of the molecules, which reduces electrostatic interactions between TNF-BP molecules and PEG-PLGA-NPs (Luey et al., 1991; Olivier et al., 1995). Both the pH and the ionic strength of incubation medium affected the amount of peptide loaded on the nanoparticles.

3.2.6. Morphology and storage stability of optimized PEG-PLGA-NPs

On the basis of the above results, we concluded that the particle size and zeta potential could be controlled by altering several experimental conditions such as the type of emulsifier, concentration of emulsifier and polymer. Furthermore, the preparation parameters, TNF-BP concentration, the pH and the ionic strength of incubation medium are important factors to affect the loading capacity of nanoparticles. The optimal conditions for preparation of nanoparticles were as follows: (1) the concentration of copolymer in the organic phase was 8.0 mg/ml. (2) The volume ratio of organic phase and aqueous phase (0.20% Tween 80) was 1:6. (3) The optimal loading of nanoparticles with TNF-BP was reached by an incubation of 40 mg blank nanoparticles with 2.0 ml TNF-BP (0.8 mg/ml) in distilled water for 12 h at 4 °C. The morphology of the optimized PEG-PLGA-NPs examined by TEM was shown in Fig. 5. It appeared that

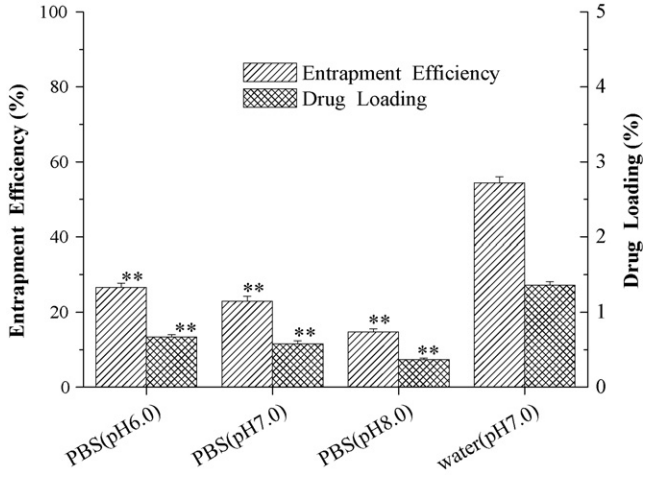

Fig. 4. Entrapment efficiency and drug loading of TNF-BP on PEG-PLGA-NPs in different incubation medium. The results were expressed as mean ± S.D. ($n = 3$). **$P < 0.01$ compared with the aqueous solution of distilled water.

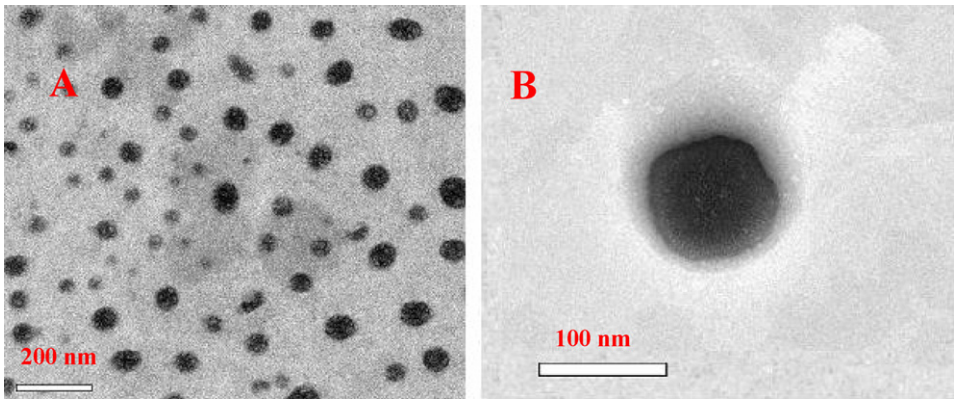

Fig. 5. (A) TEM image of PEG-PLGA-NPs (the scale bar is 200 nm). (B)TEM image of single PEG-PLGA-NPs (the scale bar is 100 nm).

the nanoparticles were regular spherical in shape without any noticeable cracks and aggregation. Average size of nanoparticles was found around 80 nm. For the stability investigations, PEG-PLGA-NPs were stored in purified water at 4 °C. As shown in Fig. 6, particle size and zeta potential did not change significantly over a storage period of 4 weeks. So the nanoparticle system was highly stable within a month.

3.3. Cellular uptake of PEG-PLGA-NPs

The aim of PLGA-NPs modified with PEG is to reduce uptake by phagocytes. To confirm the escape of PEG-PLGA-NPs from phagocytosis by phagocytes compared with PLGA-NPs, PEG-PLGA-NPs and PLGA-NPs were prepared under identical conditions (Table 5), and the nanoparticles labelled GFP uptake by MPM were analyzed by flow cytometry. As shown in Fig. 7, the data demonstrated that blank PLGA-NPs had about 4.5-folds greater uptake compared to that of blank PEG-PLGA-NPs. Indicating that the nanoparticles modified with PEG could reduce the uptake by MPM, which was in accord with the results reported previously by Fontana et al. (2001).

It was found that (Table 5) the zeta potential of PEG-PLGA-NPs was lower than that of PLGA-NPs. Previous study (Rosera et al., 1998) has shown that a lower charge of nanoparticle surfaces significantly reduced the uptake by macrophages. However, the poor surface charge might cause some problems, such as the aggregation of nanoparticles and the low amount of TNF-BP adsorbed to nanoparticle. Furthermore, it could be anticipated that the variance of phagocytosis after TNF-BP adsorbed PEG-PLGA-NPs. Owing to a charge shielding effect, the zeta potential of PEG-PLGA-NPs was decreased by loading of TNF-BP on the surface of the nanoparticles (data not shown), which consequently reduced the uptake by MPM. It was reported that the uptake of nanoparticles by MPM also declined gradually along with the increase in adsorption of hydrophilic molecules on the surface of the nanoparticles (Zhang et al., 1998). Because of some NH_2 and COOH groups, TNF-BP is a typical hydrophilic molecule. So TNF-BP adsorption might create a more hydrophilic particle surface and reduce the uptake of nanoparticles by MPM.

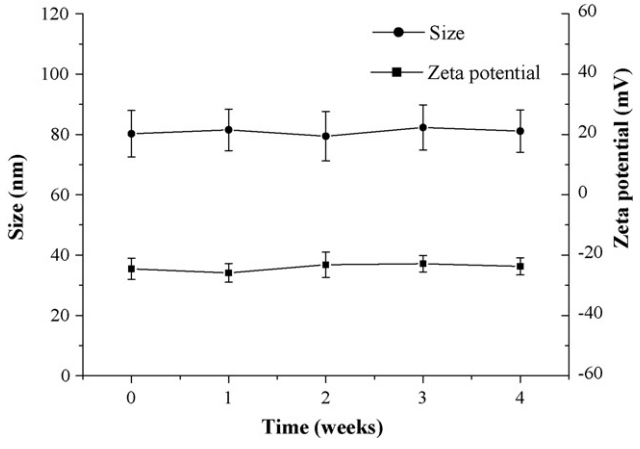

Fig. 6. Storage stability of PEG-PLGA-NPs. The results were expressed as mean ± S.D. ($n = 3$). All data were no significance.

3.4. In vitro release experiment

The TNF-BP release profiles from PEG-PLGA-NPs were shown in Fig. 8. At the identical incubation medium, the more the peptide was loaded on the nanoparticles, the easier the peptides released *in vitro*. The release of PEG-PLGA-NPs containing 0.76% TNF-BP was 70.86 ± 5.3% for 16 h, and nanoparticles containing 1.89% TNF-BP released 92.27 ± 2.5% over a period of 16 h. When the same drug loaded nanoparticles (1.89% TNF-BP) was incubated in various ionic strengths of medium for 24 h, more than 90% of peptide was released from nanoparticles in 0.20 M PBS (pH 7.4), but only 19.9 ± 3.8% of peptide was released from nanoparticles in dis-

Table 5
The characteristics of PEG-PLGA-NPs and PLGA-NPs

Copolymer	Particle size (nm)	Polydispersity index	Zeta potential (mV)
PEG-PLGA	80.2 ± 7.7	0.091 ± 0.009	−24.55 ± 3.50
PLGA	112.8 ± 11.2*	0.064 ± 0.013*	−31.28 ± 4.70

Aqueous phase (0.2% emulsifier, w/v, 30 ml), the other preparation conditions see Table 2. The results were expressed as mean ± S.D. ($n = 3$). *$P < 0.05$ compared with PLGA.

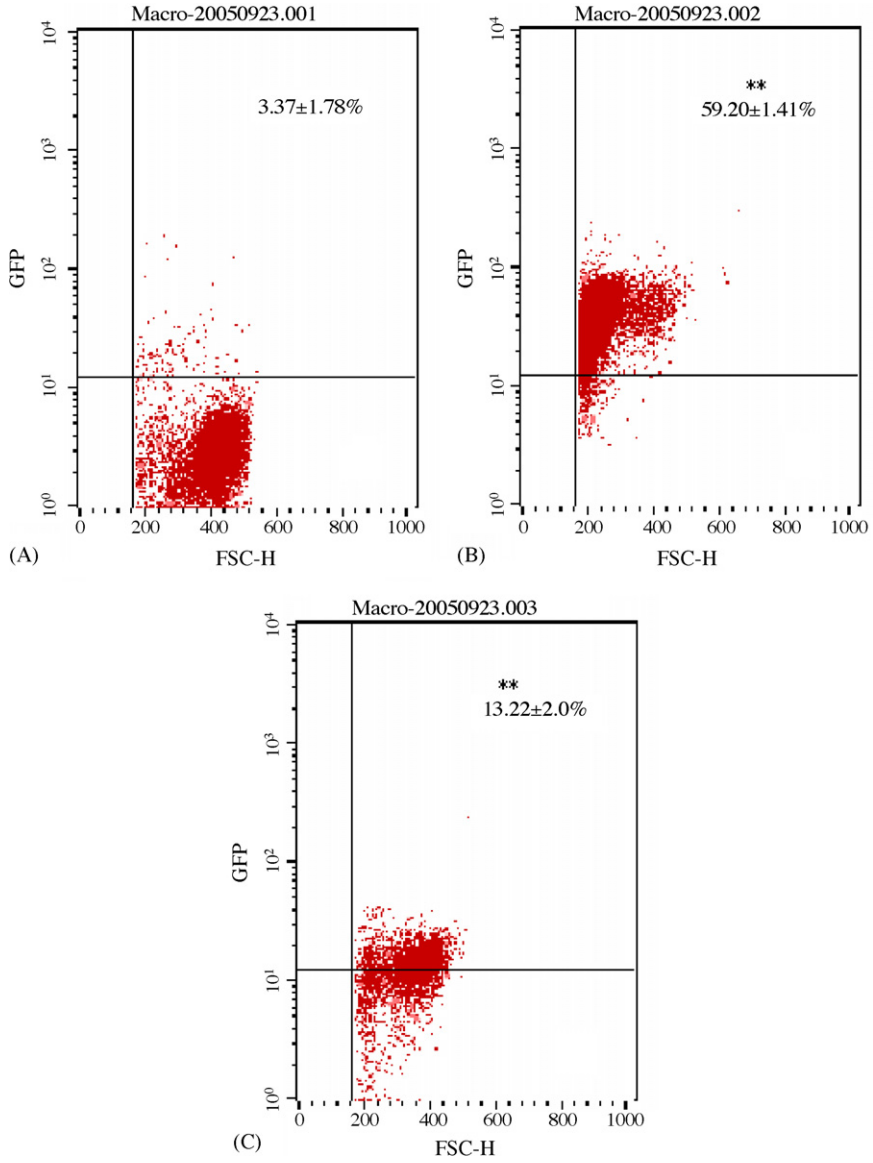

Fig. 7. The nanoparticles uptake by MPM determined by flow cytometry, MPM alone (A, control), PLGA-NPs (B) and PEG-PLGA-NPs (C). Results were expressed as a percentage of nanoparticles labelled GFP obtained from the cell of flow cytometry (mean ± S.D., $n = 3$; $^{**}P < 0.01$ compared with control).

tilled water. The difference could be explained by the fact that the high ionic strength shielded the charges of the molecules, as a consequence, the adsorbed peptide released from the nanoparticles.

The *in vitro* release results showed that the peptide released faster than that of the peptide encapsulated into those nanoparticles prepared by the W/O/W double emulsion method, which was probably attributed to the TNF-BP adsorbed to the surface of nanoparticles by electrostatic interactions. The pattern of the faster release may be useful for treatment of acute diseases, such as endotoxic shock and multi-organ function failure.

3.5. Evaluation of the bioactivity of TNF-BP released from PEG-PLGA-NPs

The major problem is that free TNF-BP is a small molecule and circulates in blood only for several minutes. The *in vitro* release experiment above suggested PEG-PLGA-NPs had the ability to sustain the release of peptides. The stability and biological activity of TNF-BP released should be also considered for the application of peptides loaded nanoparticles in human. Activity of TNF-BP released (1.89% loading, 0.20 M PBS, pH 7.4) was shown in Fig. 9. Compared with free TNF-BP, the activity of TNF-BP released for 8 h and 16 h was 56.92 ± 4.71 and 60.20 ± 3.34%, respectively. Furthermore, we compared the stability of TNF-BP in nanoparticle suspension with TNF-BP in buffer solution at 4 °C, TNF-BP in nanoparticle suspension remained active throughout the incubation period of 10 days, whereas the free peptides in buffer solution remained their activity only for 5 days (data not shown). So we presumed that PEG-PLGA-NPs may have the protective effect on TNF-PB.

In their native state, peptides possess their biologic activity. Peptides exposed to harmful conditions in nanoparticle system,

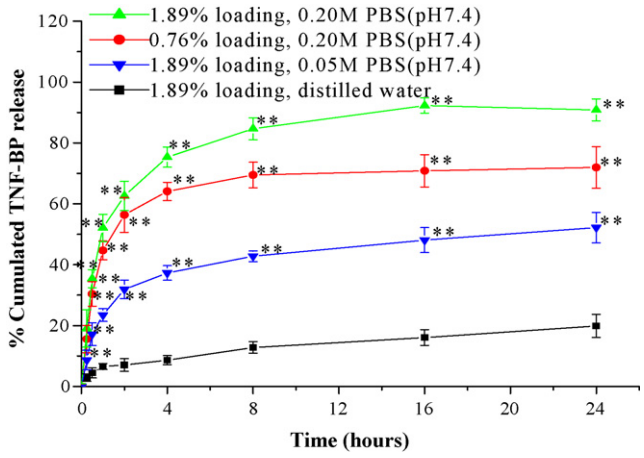

Fig. 8. *In vitro* release profiles of TNF-BP from PEG-PLGA-NPs. The results were expressed as mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared with 1.89% loading in distilled water.

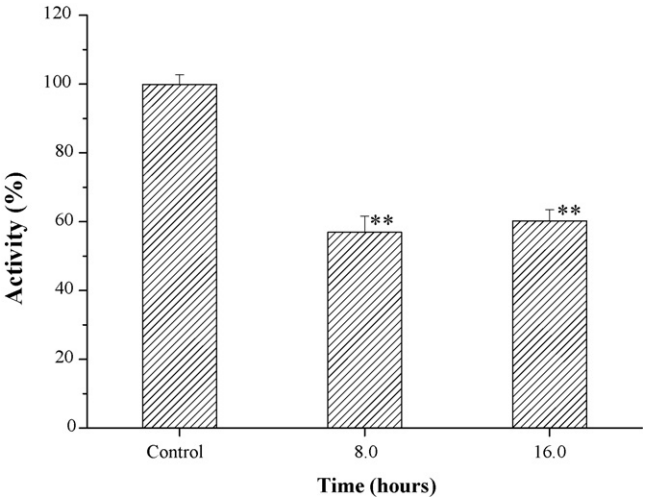

Fig. 9. Activity of TNF-BP released from PEG-PLGA-NPs in phosphate buffer solution at 37 °C. The control was the activity of TNF-BP before entrapped in nanoparticles. The results were expressed as mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared with control.

such as contact with hydrophobic surfaces and residual emulsifier, which might lead to a loss of biologic activity (Gaspar et al., 1998; Cegnar et al., 2004; Sanchez et al., 2003). In this study, TNF-BP was loaded following the preparation of blank PEG-PLGA-NPs by the modified-SESD method, which avoided the contact of TNF-BP with organic solvents during the nanoparticle preparation process. It was confirmed that the loading of TNF-BP after preparation of PEG-PLGA-NPs did less loss of TNF-BP bioactivity.

4. Conclusion

In this investigation, PEG-PLGA was synthesized by ring-opening polymerization. The structure of PEG-PLGA copolymer was confirmed by the FT-IR and ^{1}H NMR spectrum. The M_W and molecular weight distribution of the copolymers was determined by GPC. The blank PEG-PLGA-NPs were prepared by the modified-SESD method, and the physicochemical characterization of PEG-PLGA-NPs included an investigation of their properties, such as surface charge, size and morphology. The images of TEM indicated that the optimized nanoparticles were spherical in shape with smooth surface and without any aggregation or adhesion. More detailed studies showed that the particle size, zeta potential, TNF-BP concentration, pH and the ionic strength were important factors to affect the loading capacity of PEG-PLGA-NPs. By flow cytometry analysis, the uptake of PEG modified nanoparticles by MPM was found to be decreased compared with unmodified nanoparticles. *In vitro* release studies it was found that the more drugs were loaded on nanoparticles, the easier the peptides released. The peptide release became slower in lower ionic strength medium. Most of TNF-BP remained activity following release in phosphate buffer solution, and PEG-PLGA-NPs were confirmed to have the protective effect on TNF-BP. Taken together, these results suggested that PEG-PLGA-NPs could be used to load TNF-BP. However, it is also need to confirm the behavior of drug release, the speed of clearance and final biodistribution of nanoparticles *in vivo*. These issues will be pursued in our future work.

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